VIROLOGY 212, 422–428 (1995)

The Influenza Virus NS1 Protein Forms Multimers *in Vitro* and *in Vivo*

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Received May 2, 1995; accepted July 11, 1995

The NS1 protein of the influenza A virus inhibits both the nuclear export of mRNA and pre-mRNA splicing. Two functional domains, an RNA-binding domain and an effector domain, have been identified in this protein. Here we demonstrate that the NS1 protein exists as a dimer *in vitro* both in the absence of its RNA target and when it is bound to a specific RNA target, U6 snRNA. This indicates that it is most likely the dimer that binds to the RNA target. Mutational analysis indicated that the RNA-binding and dimerization domains are coincident. Multimerization also occurs *in vivo*, as assayed using the yeast two-hybrid system. In contrast to the situation *in vitro*, multimerization *in vivo* was mediated by not only the RNA-binding domain but also the effector domain. This suggests that multimerization *in vivo* involves a cellular protein cofactor that bridges more than one NS1 protein molecule together via their effector domains.

INTRODUCTION

The NS1 protein of the influenza A virus is an RNA-binding protein that regulates two posttranscriptional steps: it inhibits both the nuclear export of mRNA and pre-mRNA splicing (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Lu *et al.*, 1994; Qian *et al.*, 1994; Qiu and Krug, 1994; Qiu *et al.*, 1995). Gel shift assays demonstrated that the NS1 protein binds to poly(A) *in vitro*, and the protein inhibits the nuclear export of mRNAs containing poly(A) 3′ ends (Qiu and Krug, 1994). In addition, the NS1 protein binds to a specific stem-bulge in the spliceosomal U6 snRNA (Qiu *et al.*, 1995) and inhibits pre-mRNA splicing *in vitro* and *in vivo* (Fortes *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995). Mutational analysis identified two functional domains in the NS1 protein: an RNA-binding domain near the amino end and an effector domain in the carboxy half (Qian *et al.*, 1994). It has been presumed that the role of the effector domain is to interact with the host cell nuclear proteins needed for NS1 posttranscriptional functions.

The Rev protein of human immunodeficiency virus 1 (HIV1) also regulates nuclear RNA export, but in a way different from that of the NS1 protein. The Rev protein facilitates the nuclear export of HIV unspliced and partially spliced pre-mRNAs (Hadzopoulou-Cladaras *et al.*, 1989; Malim *et al.*, 1989a). This protein binds to a complex RNA stem–loop structure, called the Rev response element, present in HIV pre-mRNAs (Daly *et al.*, 1989; Heaphy *et al.*, 1990; Malim *et al.*, 1990; Zapp and Green, 1989). Similar to the NS1 protein, the Rev protein has primarily two domains: an RNA-binding domain and an effector domain (Malim *et al.*, 1989b). The RNA-binding domain and the sequences immediately adjacent to this domain are required for the *in vitro* multimerization of the Rev protein, resulting in the formation of primarily tetramers (Olsen *et al.*, 1990; Zapp *et al.*, 1991). However, the sequence requirements for multimerization *in vivo* are apparently different. In particular, the effector domain appears to be most critical for multimerization *in vivo* (Bogerd and Greene, 1993; Madore *et al.*, 1994). On the basis of these results, two groups have proposed that multimerization *in vivo* is mediated by a cellular protein that binds to the effector domains of more than one Rev protein.

Here we demonstrate that the NS1 protein also multimerizes. It exists as a dimer *in vitro* both in the absence of its RNA target and when it is bound to U6 snRNA. Analysis of NS1 protein mutants indicates that the RNA-binding domain and the dimerization domain of the protein are most likely coincident. Multimerization *in vivo* was found to have different sequence requirements, analogous to the situation with the Rev protein. The effector domain as well as the RNA-binding domain mediated multimerization *in vivo*, suggesting that this multimerization also involves a cellular cofactor interacting with the effector domains of more than one NS1 protein molecule.

MATERIALS AND METHODS

Purification of the GST-NS1 fusion protein

NS1 wild-type protein was expressed as a glutathione S-transferase (GST)-NS1 fusion and was purified as previously described (Qiu and Krug, 1994). Except where indicated, the NS1 protein was cleaved from the fusion by treatment with protease factor Xa. The cleaved protein was analyzed for purity by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

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0042-6822/95 $12.00

Gel filtration chromatography

One hundred micrograms of purified NS1 protein in 0.5 ml buffer A (20 m*M* HEPES, pH 7.6, 200 m*M* NaCl, 20% glycerol) was loaded onto a 1-cm-diameter column packed with 28 ml of Sephacryl S-200 Super Fine in buffer A. One-milliliter fractions were collected, and 50-μl aliquots of each fraction were electrophoresed on a 14% SDS–polyacrylamide gel. The gel was transfered to a nitrocellulose membrane (Schleicher & Schuell) using a Pharmacia LKB-Multiphor II electroblotter, and the NS1 protein was detected by immunoblotting (Amersham ECL Western Blotting kit) using NS1 monoclonal antisera (kindly provided by Jonathan Yewdell).

Chemical crosslinking

Purified NS1 protein (200 ng) was incubated with glutaraldehyde (Fisher Scientific) in buffer A in 20-μl reaction volumes and incubated for 30 min at 25°. Samples were electrophoresed on a 14% SDS–polyacrylamide gel which was subsequently transferred to a nitrocellulose membrane that was then probed with NS1 monoclonal antisera.

In vitro translation of NS1 proteins

NS1 wild-type and mutant DNAs (Qian *et al.*, 1994) were subcloned into the *in vitro* transcription vector pGEM1 and translated using a Promega TnT Coupled Transcription/Translation kit and [^{35}S]L-methionine (800 Ci/mmole; NEN).

Glutathione Sepharose affinity selection

Purified GST-NS1 wild-type protein (1 μg) was combined with 5 μl of *in vitro* translated ^{35}S-labeled NS1 mutant (or wild-type) protein, 20 μl of glutathione Sepharose 4B beads (Pharmacia), and 0.2 ml of IPP_{150} buffer (10 m*M* Tris–HCl, pH 8.0, 0.15 *M* NaCl, and 0.1% Nonidet P-40) in 1.5-ml microcentrifuge tubes. The tubes were incubated at 4° for 1.5 hr with constant rotation. The beads were washed three times with 1 ml of IPP_{150} buffer, followed by a wash in IPP_{375} buffer (same as IPP_{150} buffer but with 0.375 *M* NaCl). The beads were resuspended in gel loading buffer containing 5% (v/v) 2-mercaptoethanol and heated at 95° for 2 min. The resulting eluate was analyzed by electrophoresis on a 14% SDS–polyacrylamide gel. The ^{35}S-labeled NS1 proteins were detected by fluorography of the SDS–polyacrylamide gel.

Biotin–streptavidin affinity selection

Full-length U6 snRNA and U6 snRNA lacking the first 40 nucleotides (Δ 1–40) were synthesized in the presence of 0.4 m*M* biotin-21-UTP (Clontech Laboratories) using T7 RNA polymerase. Biotin-labeled U6 snRNA (200 ng) was incubated with 1 μg of purified NS1 protein in a 20-μl reaction volume containing KHN buffer (150 m*M* KCl, 20 m*M* HEPES, pH 7.6, 0.05% Nonidet P-40, 0.2 m*M* dithiothreitol, 0.5 m*M* phenylmethylsulfonyl fluoride) (Boelens *et al.*, 1993). Where indicated, the RNA–protein complexes were treated with 0.01% glutaraldehyde at this point for 30 min at room temperature. Each reaction mixture was added to 20 μl of streptavidin agarose beads (BRL) and 0.5 ml of KHN buffer and was then incubated at room temperature for 60 min with constant rotation. The beads were washed five times with 0.5 ml of KHN buffer, resuspended in gel loading buffer containing 5% (v/v) 2-mercaptoethanol, and boiled 2 min. The eluates were electrophoresed on a 14% SDS–polyacrylamide gel which was then transferred to a nitrocellulose membrane that was then probed for NS1 protein using NS1-specific antisera as described above.

Yeast two-hybrid assays

The complete NS1 open reading frame of wild-type and mutant NS1 DNAs was inserted into yeast vectors pGAD2F and pMA424. The former vector expressed a fusion between the DNA-binding domain of Gal4 and the NS1 protein. The latter vector expressed a fusion between the activation domain of Gal4 and the NS1 protein. These plasmids, which contain a HIS3 and a LEU2 marker, respectively, were cotransformed into yeast strain GGY::171, which carries the Gal1-lacZ reporter gene. As a positive control for two-hybrid interaction, yeast strain GGY::171 was transformed with plasmids expressing the known interacting proteins SNF1 and SNF4 as Gal4 fusions. Transformants were plated on yeast media lacking histidine and leucine. Primary transformants were screened for β-galactosidase expression by replica plating assays using X-gal as a chromogenic indicator. The color of the colonies was determined 30 min after the addition of X-gal. Quantitative β-galactosidase activities were determined by growing transformants to a midlogarithmic phase in synthetic complete media containing 2% galactose, 2% glycerol, and 2% ethanol and lacking histidine and leucine to select for the plasmids (Miller, 1972).

RESULTS

The NS1 protein exists as a dimer *in vitro* before binding to its RNA target

To determine the oligomerization state of the NS1 protein in the absence of its RNA target, the NS1 protein was analyzed by gel filtration chromatography on Sephacryl S-200 (Fig. 1A). Little or none of the NS1 protein eluted at the position expected for an NS1 protein monomer (approximately 25K). Instead, the peak fraction of the NS1 protein eluted at the position expected for a dimer of the NS1 protein (approximately 50K), indicating that almost all of the NS1 protein existed as a dimer *in vitro*.

To verify this result, the NS1 protein was exposed to various levels of the crosslinking reagent glutaraldehyde, and the reactions were analyzed by electrophoresis on

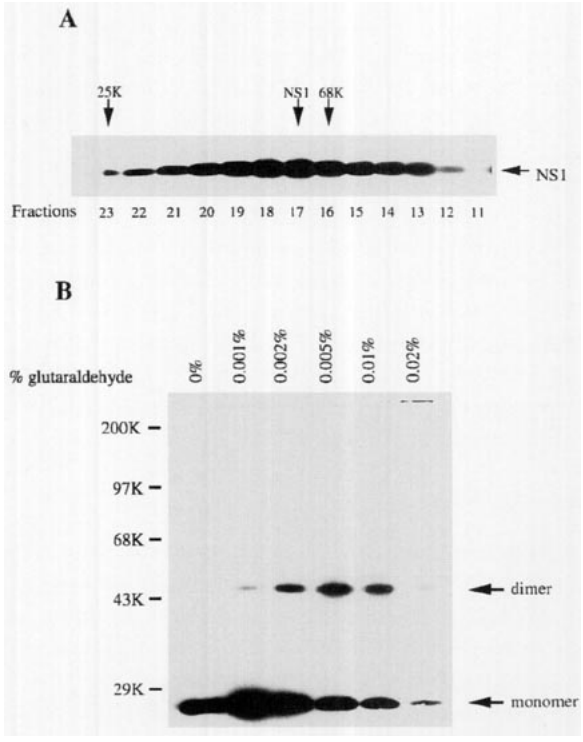

FIG. 1. The NS1 protein exists as a dimer in vitro in the absence of an RNA target. (A) Chromatography of the NS1 protein on a Sephacryl S-200 Super Fine column. One-milliliter aliquots of each column fraction were assayed for the NS1 protein by immunoblotting using anti-NS1 antisera. The peak column fraction of the NS1 protein is indicated by an arrow, as are the peak fractions of the globular molecular weight standards ribonuclease A (25K) and bovine serum albumin (68K) run previously on the same column. (B) Chemical crosslinking of the NS1 protein. Purified NS1 protein was treated with glutaraldehyde at the final concentration (v/v) indicated. The samples were resolved on a 14% SDS–polyacrylamide gel, and the NS1 protein was detected by immunoblotting with anti-NS1 antisera. The positions of the NS1 monomer and dimer forms are indicated by arrows. Protein molecular weight standards are indicated.

a denaturing gel (Fig. 1B). After glutaraldehyde treatment, a significant fraction of the NS1 protein migrated as a dimer. The most efficient formation of dimer occurred with 0.005% glutaraldehyde. About 30% of the NS1 protein migrated as a dimer, indicating that glutaraldehyde crosslinking was only about 30% efficient. Multimers larger than dimers were not observed. These results indicate that the NS1 protein exists as a dimer *in vitro*.

Mapping the *in vitro* dimerization domain of the NS1 protein

Two functional domains have been identified in the NS1 protein: an RNA-binding domain and an effector domain (Qian *et al.*, 1994) (Fig. 2B). We devised an assay to locate the domain required for dimerization. A GST-NS1 fusion protein was bound to glutathione agarose beads, and the ability of an ^{35}S-labeled nonfusion NS1 protein to complex with the bound GST-NS1 fusion protein was determined (Fig. 2A). In the results shown, wild-type NS1 protein was part of the GST-NS1 fusion, and the ^{35}S-labeled nonfusion NS1 protein was either wild type or mutant. In other experiments, GST-NS1 fusions containing mutant NS1 proteins were used in combination with ^{35}S-labeled nonfusion wild-type or mutant NS1 proteins; the same results were obtained.

The only mutated NS1 proteins that failed to complex to the wild-type NS1 protein were those that contained deletions of, or point mutations in, the RNA-binding domain, namely, Δ1, M2, M3, and M4 (Fig. 2C). The lack of dimerization of the M3 mutant protein was confirmed by a glutaraldehyde crosslinking experiment (data not shown). The boundaries of the dimerization domain were fairly well defined by two mutants that are wild type in RNA binding and in the inhibition of mRNA export and pre-mRNA splicing *in vivo* (Qian *et al.*, 1994; Lu *et al.*, 1994): mutant M1, which contains a mutation upstream from the amino end of the RNA binding domain, and mutant M5, which contains a mutation immediately adjacent to the carboxy end of the RNA-binding domain. Both of these mutants retained high activity in binding to the wild-type NS1 protein. These results strongly suggest that the dimerization domain of the NS1 protein is coincident with its RNA-binding domain.

The NS1 protein bound to its RNA target is also in the form of a dimer

The NS1 protein has been shown to bind to a specific stem-bulge in U6 snRNA (Qiu *et al.*, 1995). We determined whether the NS1 protein bound to U6 snRNA was also in the form of a dimer, using the experimental design diagramed in Fig. 3A. The NS1 protein was incubated with full-length U6 snRNA containing biotinylated uridine residues, and the U6 snRNA was then affinity selected using streptavidin agarose beads. The NS1 protein bound to the full-length U6 snRNA was isolated by this procedure (Fig. 3B, lane 2). The specificity of this selection procedure was established using a U6 snRNA lacking nucleotides 1–40 (Δ 1–40), which has been shown not to bind to the NS1 protein (Qiu *et al.*, 1995): little NS1 protein was selected using this truncated U6 snRNA (lane 4). To determine the oligomerization state of the NS1 protein bound to the full-length U6 snRNA, the affinity-selected protein was treated with glutaraldehyde, prior to electrophoresis on a denaturing gel (lane 3). About 15% of the bound NS1 protein migrated as a dimer. Because the efficiency of glutaraldehyde crosslinking was about 30%, these results indicate that about 50% of the NS1 protein bound to U6 snRNA was in the form of a dimer.

The NS1 protein also multimerizes *in vivo*, but with different sequence requirements

The *in vivo* multimerization of the NS1 protein was analyzed using the yeast two-hybrid system. In this assay system, when a protein fused to the DNA-binding domain of the Gal4 transcription factor interacts with a protein

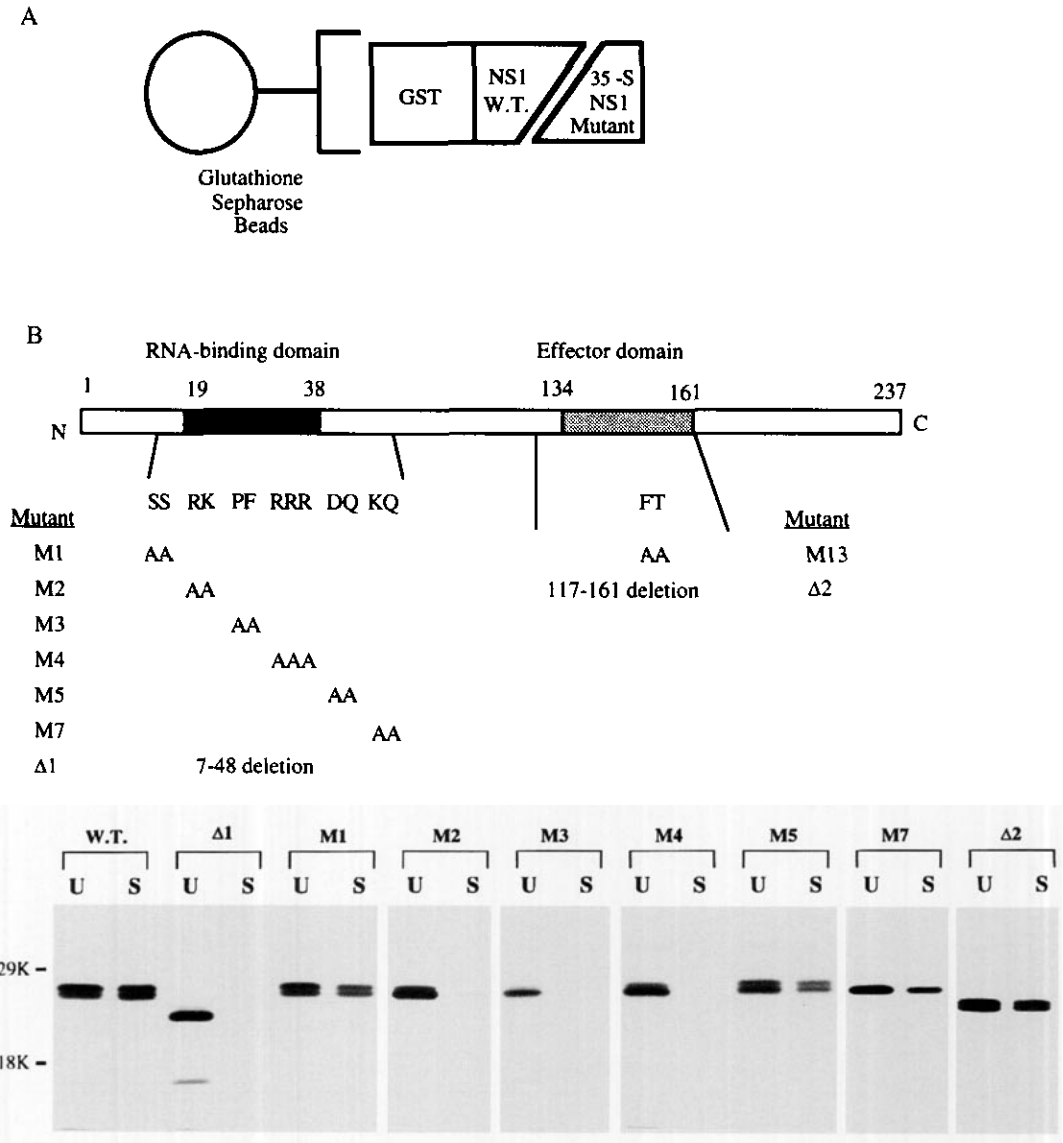

FIG. 2. Identification of the NS1 multimerization domain by a glutathione selection assay. (A) Schematic diagram of assay. (B) Description of the NS1 mutants characterized. The mutants to the left have the indicated mutation or deletion in or near the RNA-binding domain, and the mutants to the right have the indicated mutation or deletion in the effector domain. It should be noted that the M1, M5, and M7 mutants, which have mutations outside the RNA-binding domain, are wild type in RNA-binding *in vitro* and in the inhibition of mRNA export and pre-mRNA splicing *in vivo* (Qian *et al.*, 1994; Lu *et al.*, 1994). (C) Binding of ^{35}S-labeled NS1 mutant or wild-type proteins to purified GST-NS1 wild-type protein. Five microliters of each ^{35}S-labeled NS1 mutant or wild-type protein was incubated with 1 μg of purified GST-NS1 protein and 20 μl of glutathione Sepharose 4B beads. The ^{35}S-labeled NS1 protein eluted from the beads was analyzed by electrophoresis on a 14% SDS–polyacrylamide gel: S, selected protein; U, the unselected NS1 protein, i.e., the NS1 protein prior to selection. Protein molecular weight standards are indicated.

fused to the Gal4 activation domain, the *lac Z* reporter gene is activated so that the resulting β-galactosidase-producing colonies turn blue in the presence of X-gal as a chromogenic indicator (Fields and Song, 1989). Using this assay, the interaction of wild-type NS1 protein with itself was evident by the appearance of blue colonies (Table 1). The level of β-galactosidase expression was high, about five times that observed for the positive control, the interaction of SNF1 and SNF4, which provided the prototype of the two-hybrid system (Fields and Song, 1989). NS1 proteins containing either a mutation in the RNA-binding domain or a deletion of the RNA-binding domain (Δ1) retained the ability to interact with themselves, as evidenced by the blue color of the colonies. Though β-galactosidase activity was significantly reduced, the remaining activity was comparable to that of the SNF1–SNF4 positive control. These results indicate that in the absence of a functional RNA-binding domain, the interaction between NS1 proteins remained but the strength of this interaction was reduced. Hence, unlike the situation *in vitro*, the RNA-binding domain was not solely responsible for the multimerization of the NS1 protein *in vivo*.

To identify the domain(s) participating in multimeriza-

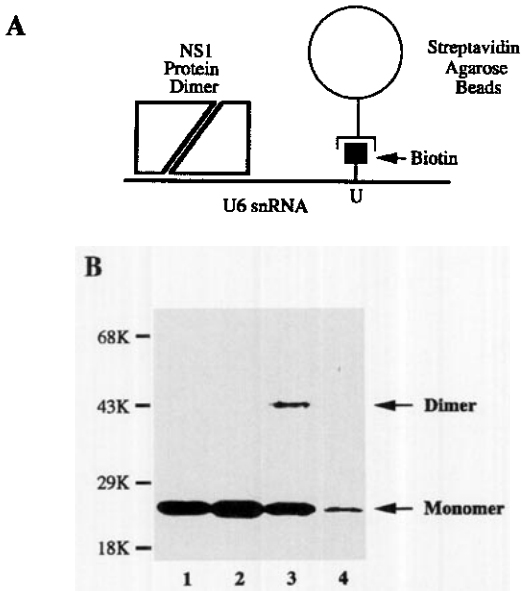

FIG. 3. The NS1 protein bound to U6 snRNA is in the form of a dimer. (A) Schematic diagram of the isolation of NS1 protein bound to U6 snRNA. (B) Purified NS1 protein was incubated with biotinylated full-length U6 snRNA (lanes 2 and 3) or truncated U6 (Δ1–40) snRNA (lane 4), and the NS1 protein bound to the U6 snRNA was isolated by affinity chromatography to streptavidin agarose beads as depicted in A. One of the NS1 protein–full-length U6 snRNA mixtures was then treated with glutaraldehyde (lane 3). The proteins were analyzed by gel electrophoresis, and the NS1 protein was detected by immunoblotting. Lane 1, untreated NS1 protein. Protein molecular weight standards are indicated.

tion *in vivo*, other mutations or deletions were made. A mutation (M13) or a deletion (Δ2) of the effector domain, each of which causes a loss of function in mRNA export and pre-mRNA splicing *in vivo* (Qian *et al.*, 1994; Lu *et al.*, 1994), did not eliminate the *in vivo* interaction between NS1 protein molecules, as shown by the blue colonies. β-galactosidase activity was reduced to a level comparable to, or slightly greater than, the level observed with the SNF1–SNF4 positive control. This indicated that the NS1 protein interactions remaining in the absence of a functional effector domain were not as strong as those occurring with wild-type NS1 proteins. Only after inactivating both the RNA-binding domain and the effector domain, either by mutations (M3 and M13) or by deletions (Δ1 and Δ2), did the NS1 protein lose its ability to interact with itself. This loss of activity was not due to the presence of insufficient NS1 protein, as Western analysis indicated that the amount of NS1 protein produced in a white yeast colony (e.g., the M3 plus M13 colonies) was equivalent to that produced in a blue yeast colony (e.g., the M3 colonies) (Fig. 4). Hence, the interaction of the NS1 protein with itself *in vivo* most likely involves both the RNA-binding domain and the effector domain.

DISCUSSION

Our results indicate that the NS1 protein forms multimers both *in vitro* and *in vivo* and that the sequence requirements for this multimerization are different *in vitro*

TABLE 1

Analysis of NS1 Protein Multimerization *in Vivo*

NS1 protein fused to		Colony color	β-Galactosidase activity
Binding domain	Activation domain		
Wild type	None	White	<1
None	Wild type	White	<1
Wild type	Wild type	Blue	102
Mutant 3	Mutant 3	Blue	25
Δ1	Δ1	Blue	18
Mutant 13	Mutant 13	Blue	21
Δ2	Δ2	Blue	33
Δ1, Δ2	Δ1, Δ2	White	<1
Δ1, mutant 13	Δ1, mutant 13	White	<1
Mutant 3, Δ2	Mutant 3, Δ2	White	<1
Mutant 3, mutant 13	Mutant 3, mutant 13	White	<1
Other proteins fused to			
Binding domain	Activation domain		
SNF1	SNF4	Blue	19

Note. Yeast two-hybrid analysis of the NS1 protein. NS1 constructs in the Gal4 activation domain vector (pGAD2F) and the Gal4 DNA-binding domain vector (pMA424) were transformed into yeast Gal1 reporter strain GGY::171, and the transformants were assayed for β-galactosidase activity (see Materials and Methods). Interacting hybrid proteins (positive for β-galactosidase activity) are indicated by blue colonies. Noninteracting hybrid proteins are indicated by white colonies. All mutants listed are described in *Fig. 2B*. Quantitative β-galactosidase activities of liquid cultures are given in Miller units (Miller, 1972). Values >1 are averages of three independent transformants with standard errors <10%.

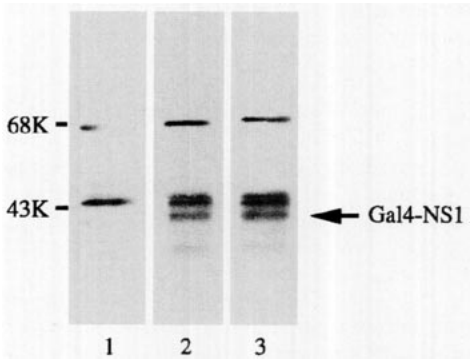

FIG. 4. Similar levels of the NS1 protein were produced in white (−) and blue (+) yeast colonies. Yeast colonies transformed with the NS1 mutant constructs in the Gal4 DNA-binding domain vector (pMA424) were grown in liquid culture, and protein lysates were analyzed for NS1 protein expression by immunoblotting with NS1-specific antisera. Lane 1, pMA424 (no NS1 sequences). Lane 2, NS1 M3 mutant fused to pMA424. Lane 3, NS1 M3, M13 double mutant fused to pMA424.

and *in vivo*. For multimerization *in vitro* only the RNA-binding domain was involved, whereas for multimerization *in vivo* both the RNA-binding domain and the effector domain participated. We have shown that the *in vitro* multimer is a dimer, but the stoichiometry of the *in vivo* multimer has not been established.

We demonstrated that the NS1 protein exists as a dimer *in vitro* both in the absence of its RNA target and when it is bound to its U6 snRNA target. This indicates that it is most likely the dimer, and not the monomer, that binds to U6 snRNA. In fact, it can be postulated that only the dimer, and not the monomer, is capable of specific RNA binding. According to this hypothesis, the RNA-binding region (amino acids 19 through 38) of an NS1 protein monomer would not be able to bind to U6 snRNA. A functional RNA-binding domain would be formed only when the RNA-binding domains of two molecules of the NS1 protein interact to form a NS1 protein dimer.

It is not clear whether the HIV-1 Rev protein behaves *in vitro* in the same way as the NS1 protein. The Rev protein forms tetramers *in vitro*, but it remains to be resolved whether Rev tetramerization is required for specific RNA binding or whether multimerization occurs after the binding of a monomer to the RNA-binding site (Olsen *et al.*, 1990; Cook *et al.*, 1991; Kjems *et al.*, 1991; Malim and Cullen, 1991; Zapp *et al.*, 1991; Iwai *et al.*, 1992; Cole *et al.*, 1993; Tiley *et al.*, 1992). In addition, the Rev multimerization domain apparently includes not only the RNA-binding domain but also sequences immediately amino- or carboxy-terminal to the RNA-binding domain (Olsen *et al.*, 1990; Malim and Cullen, 1991; Zapp *et al.*, 1991). In contrast, our data indicate that the RNA-binding and multimerization domains of the NS1 protein are coincident. In particular, the M5 mutation which is immediately carboxy-terminal to the RNA-binding domain allows multimerization of the NS1 protein to occur *in vitro*.

Multimerization of the NS1 protein *in vivo* involves both its RNA-binding domain and its effector domain. In the absence of a functional RNA-binding domain, the NS1 protein still multimerizes, in contrast to the situation *in vitro*. Only when the effector domain as well as the RNA-binding domain was inactivated (by mutation or deletion) was multimerization absent. A similar role for the effector domain has been reported for the *in vivo* multimerization of the HIV Rev protein and the human T-cell leukemia virus type 1 Rex protein. On the basis of this observation, it was proposed that a cellular protein bridges Rev or Rex proteins together by binding to the effector domains of more than one Rev or Rex protein. A cellular protein may serve the same function for the NS1 protein. Because our *in vivo* assays for NS1 protein multimerization were carried out in yeast, the putative cellular bridging protein might be expected to be present in yeast cells. This suggests that some of the functions of the NS1 protein might occur in yeast as well as in mammalian cells. We are currently testing this possibility.

Based on these results, our working hypothesis is that functional NS1 proteins *in vivo* are in the form of multimers in which the component NS1 protein molecules interact with each other via two domains, the RNA-binding domain and the effector domain. The RNA-binding domain interaction would be the same as that occurring *in vitro*. As is the case *in vitro*, this interaction would occur both before and after binding of the NS1 protein to its RNA targets. It should be pointed out that at least one of the RNA targets of the NS1 protein, namely, poly(A), is present in the yeast cells used in the two hybrid assays, and it is therefore conceivable that the NS1 protein molecules in these yeast cells are bound to RNA target molecules. The effector domain interaction between NS1 protein molecules would be mediated by one or more cellular bridging proteins. It will be of great interest to identify the putative cellular proteins that bind to the effector domains of NS1 proteins.

ACKNOWLEDGMENTS

We thank Helen Pirello for help in preparing this manuscript and Brian Pollack for advice on performing β-galactosidase liquid assays. This investigation was supported by a National Institutes of Health grant (AI 11772, merit award) to R.M.K.

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